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13. ABSTRACT (Maximum 200 Words)

The primary objective of the proposed research is to investigate the role of the Gadd45 family of genes (Growth Arrest & DNA Damage) (Gadd45a and Gadd45b) in breast carcinogensis, which have been shown to play an important role in cell cycle control and response to anti-cancer agents. A research plan was designed taking advantage of established breast cancer prone mouse models (MMTV-v-Ras and MMTV-c-Myc) that were crossed with Gadd45a or Gadd45a/b deficient mice. We have successfully generated mouse strains that are deficient for Gadd45a and breast cancer prone as a result of expression of oncogenic Myc (MMTV-c-Myc Gadd45a-/-) or activated Ras (MMTV-v-Ras Gadd45a-/-). Preliminary observations suggest breast tumorigenesis is accelerated in the MMTV-v-Ras Gadd45α-/- group when compared to the MMTV-v-Ras Gadd45α+/+ group. Tumors begin to appear in the MMTV-v-Ras Gadd45α-/- mice as early as 2 months of age, whereas the wildtype mice do not seem to develop tumors until 5 months of age. Interestingly, Gadd45α+/- mice begin to develop tumors at 4 months of age, which suggests haplo-deficiency of Gadd45α is sufficient for acceleration of mammary tumor development. Another interesting observation is the increase in the number of tumors per mouse in the MMTV-v-Ras Gadd45α-/- and MMTV-v-Ras Gadd45α+/- treatment groups compared to MMTV-v-Ras Gadd45α+/+ group. Results for MMTV-c-Myc mice are in the process of being collected and evaluated.

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INTRODUCTION

The development of breast cancer is a multistage process. Alterations in multiple genes that control cell proliferation, survival and metastasis are known to cooperate in the development and progression of breast tumorigenesis. The overall objective of the proposed research is to investigate the role of the Gadd45 family of genes (Growth Arrest & DNA Damage) (Gadd45α and Gadd45β) in breast carcinogenesis, which have been shown to play an important role in cell cycle control and response to anti-cancer agents. In order to achieve this, a research plan was designed taking advantage of established breast cancer prone mouse models (MMTV-v-Ras and MMTV-c-Myc) that are being crossed with Gadd45a or Gadd45a/b deficient mice. MMTV-v-Ras mice develop spontaneous mammary adenocarcinomas beginning approximately 6 months of age, whereas MMTV-c-Myc mice develop masses beginning at approximately 9 months of age. Mating these mice with Gadd45 deficient mice, which are not prone to the development spontaneous mammary adenocarcinomas, will allow for the establishment of breast cancer prone mouse strains that are deficient for one or more Gadd45 genes, which will be used to investigate how the loss of Gadd45 may promote breast cancer formation or progression under different experimental settings (i.e. presence or absence of hormone treatment, IR treatment, or DMBA treatment). We hypothesis that the loss of Gadd45α and Gadd45α/β will accelerate tumorigenesis. Expanding the focus of the research, our experimental plans include generating tumor cell lines from primary tumor masses collected from the mice and characterizing the cellular and molecular pathways involved in breast carcinogenesis and determining the role Gadd45 plays in these processes.

BODY

Task 1. *Assess the effect of Gadd45α & Gaddα/β deficiencies on breast tumorigenesis induced by hormone, IR or chemical carcinogen using breast cancer susceptible Balb/cMed mice.*

A. Transfer Gadd45α-/- and Gadd45 α/β-/- null alleles onto the genetic background of breast cancer prone Balb/cMed mice, by backcrossing for 5 generations. **(Months 1-10)**

B. Assess the effect of Gadd45α & Gad45α/β deficiencies on breast tumorigenesis in Balb/c mice following hormone stimulation, treatment with IR or with DMBA **(Months 10-24)**

C. Cellular/molecular characterization of breast tumors promoted by Gadd45 deficiency & hormone stimulation, or treatment with IR or DMBA **(Months 20-36)**

We have recently submitted an official change in the Statement of Work (SOW) for this award. The revised SOW allows for the elimination of Task 1 (See Submitted Revised SOW-appendix 1) (Pending Approval). Originally, Task 1 and Task II overlapped in most, if not all aspects of work and share the same overall objectives. Essentially, they provided a redundancy to increase the chance of success. Over the past year, Task II has begun to provide interesting results. Thus, we believe that by focusing on and expanding Task II for the remaining of this granting period, it will enable us to address the overall objective more precisely, which will result in more significant results and improved quality of reportable outcomes.

Task 2. *Assess the effect of GADD45α & Gadd45α/β deficiencies on oncogene driven breast carcinogenesis.*

A. Establishment of MMTV-v-ras and WAP-c-myc mice that are deficient for Gadd45α or Gadd45α/β will be accomplished by matting Gadd45α-/- & Gad45α/β-/- knockout mice with MMTV-v-ras (Charles River lab) and WAP-c-myc (Jackson Lab) **(Months 1-14)**

B. Effect of Gadd45α & Gad45α/β deficiencies on breast cancer development in MMTV-v-ras and WAP-c-myc mice that are WT or deficient for Gadd45α or Gadd45α/β expression will be explored following the same path as described in AIM 1B **(Months 14-20).**

C. Cellular/molecular characterization of breast tumors promoted by Gadd45 deficiency and oncogenic ras or c-myc will be ascertained following the same path as described in AIM 1C. **(Months 20-36)**

Another revision in the SOW provided for the switch from WAP-c-Myc mice to MMTV-c-Myc mice. This was decided upon to provide a more attractive experimental model. The MMTV-c-Myc mouse model allows for the use of MMTV promoter, which is the same as the MMTV-v-Ras mouse model. Therefore, expression of the oncogenic myc and activated ras will be under the same promoter controls. This provides similar patterns and level of expression in the mammary tissue in both experimental groups. Also, it has been shown that spontaneous mammary adenocarcinomas form in MMTV-c-Myc mice starting at approximately 6-9 months, whereas in WAP-c-Myc mice develop tumors as early as 3 months. Thus, by making this change, the effects of Gadd45 deficiency on c-Myc driven breast carcinogenesis will be more pronounced and easier to study in the MMTV-c-Myc mice.

As reported in last year's annual report, we are actively establishing mouse strains that express either oncogenic Myc (MMV-c-Myc) or activated Ras (MMTV-v-Ras) that are deficient for Gadd45α or Gadd45α/β, along with appropriate control mice. During the past year, we have been generating mice that express the desired genotypes. Following proper PCR genotyping protocols (Figure included 2003 Annual Report), the mice are placed in the appropriate experimental treatment groups (i.e. No Treatment, IR Treatment) and monitored for tumor formation and progression. Table 1 is a complete list of the number of mice in each experimental treatment group. We are in the lengthy process of continuing to fill the treatment groups until all groups have a minimum of 12 mice and monitoring the mice for tumor development and progression over a 52 week period. As seen in Table 1C the MMTV-c-Myc treatment groups have not been filled as efficiently as the MMTV-v-Ras treatment groups. This is due to the unique characteristic of the MMTV-c-Myc that the females do not nurse their newborn pups. As a result, foster mothers must first be established in order for a litter to survive, which is not always guaranteed. This explains the observable lagging of the MMTV-c-Myc treatment groups being filled.

A. MMTV-v-Ras -- No Treatment

Genotype	Total	Number
Ras + G -/-	23	***588(38) 590(39) 591(26) 599(34) 600(11) 601(38) 612(49)*** 616(44) ***619(15)*** 620(41) 624(40) 641(34) 650(32) 669(26) ***670(12)*** 730(11) 731(11) ***741(8)*** 742(11) 744(11) ***745(8) 767(9) 768(9)***
Ras + G +/-	9	***614(31) 621(26) 623(43)*** 626(44) ***628(30)*** 743(11) ***747(16) 748(15) 749(25)***
Ras+ G +/+	15	***613(49) 615(48) 617(41) 618(39) 622(49) 623(49) 625(44)*** 651(36) 679(36) ***680(35) 683(24) 684(28)*** 685(36) 686(36) 729(36)

B. MMTV-v-Ras – IR Treatment -- (3 doses of 3gy every other day)

Genotype	Total	Number
Ras + G -/-	18	***604(24) 605(29) 606(29) 609(21)*** 709(18) 710(18) 711(18) 712(18) 715(15) ***716(14) 717(14)*** 718(15) 719(15) 769(9) 770(9) 771(9) 772(9) 773(9)
Ras + Gadd +/-	0	
Ras + Gadd +/+	0	

C. MMTV-c-Myc – No Treatment

Genotype	Total	Number
Myc + G -/-	4	627(44) 668(30) 744(11) 746(11)
Myc + Gadd +/-	1	628(35)
Myc + Gadd +/+	0	

Table 1:
Table 1 is a complete list of the number of mice in each of the respective treatment groups. The mouse ID number is given, followed by the age in weeks of the mouse. The regular font numbers are alive mice that are actively being monitored. The ***bold/italic*** numbers are mice that developed tumors and were euthanized to collect the tumor masses. The age in weeks of tumor onset follows.

Although rigorous quantization and definitive conclusions regarding mammary tumor incidence and age of tumor onset can not be obtained until the completion of the experimental protocol, interesting results have been observed from the preliminary data. Using the mice that have developed tumors to date from the No Treatment Ras Group, tumorigenesis seems to be accelerated in the MMTV-v-Ras Gadd45α-/- group when compared to the MMTV-v-Ras Gadd45α+/+ group (Figure 1). Tumors begin to appear in the MMTV-v-Ras Gadd45α-/- mice as early as 2 months of age, whereas the wildtype mice do not seem to develop tumors until 5 months of age (Figure 1A-B). Interestingly, Gadd45α+/- mice begin to develop tumors at 4 months of age, which suggests haplo-deficiency of Gadd45α is sufficient for acceleration of mammary tumor development (Figure 1C).

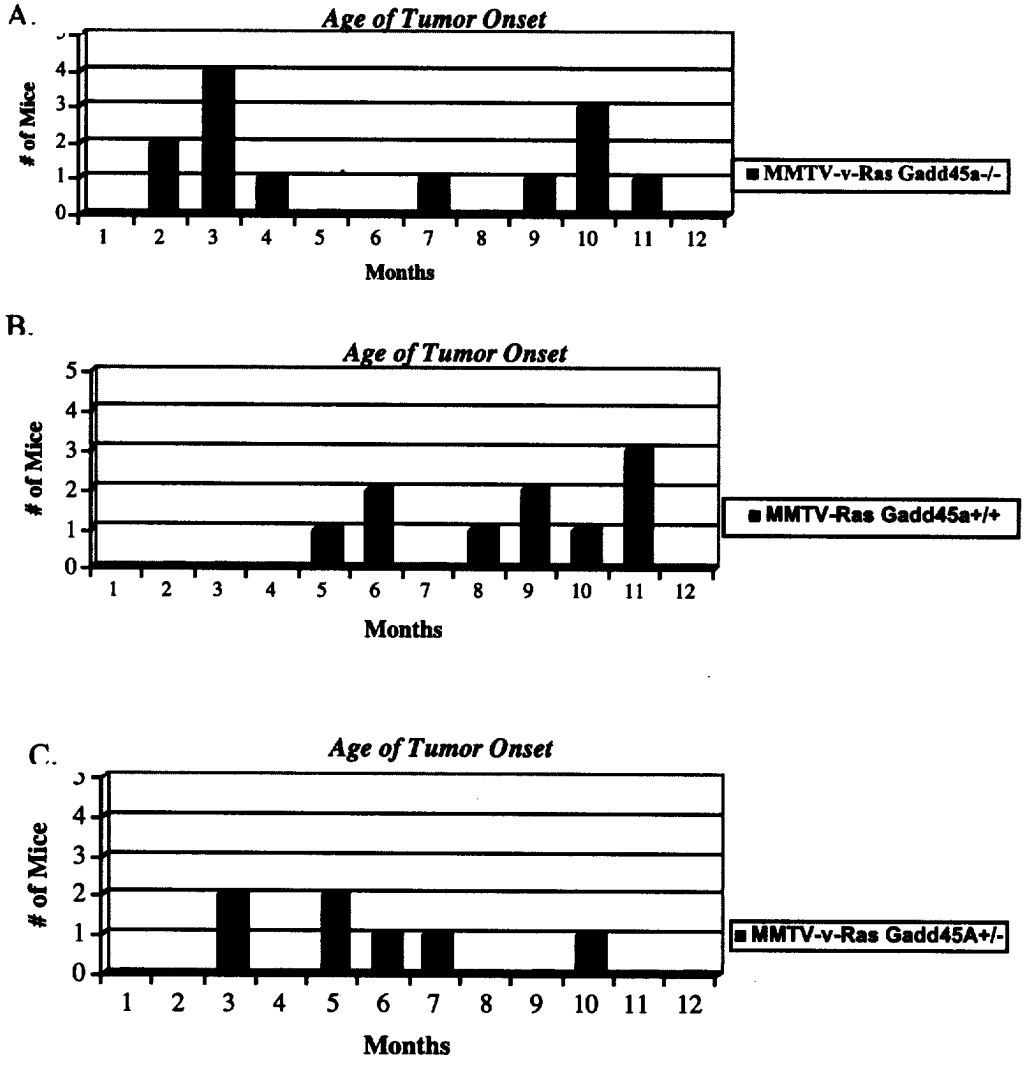

Figure 1: Age of Tumor Onset for mice expressing MMTV-v-Ras. A. Tumor onset for MMTV-v-Ras Gadd45α-/- mice begins at 2 months of age. B. Tumor onset for MMTV-v-Ras Gadd45+/+ mice begins at 5 months of age. C. Tumor onset for MMTV-v-Ras Gadd45α+/- mice begins at 3 months of age.

Using the mice that have developed tumors from the Ras IR treatment group, breast tumorigenesis seems to be accelerated when compared to unirradiated mice (Figure 2). (IR treatment = 3 doses of 3 greys). We are in the process of filling the control IR experimental groups and monitoring them for tumor development to more accurately compare these mice. Evaluation of this data is under careful review.

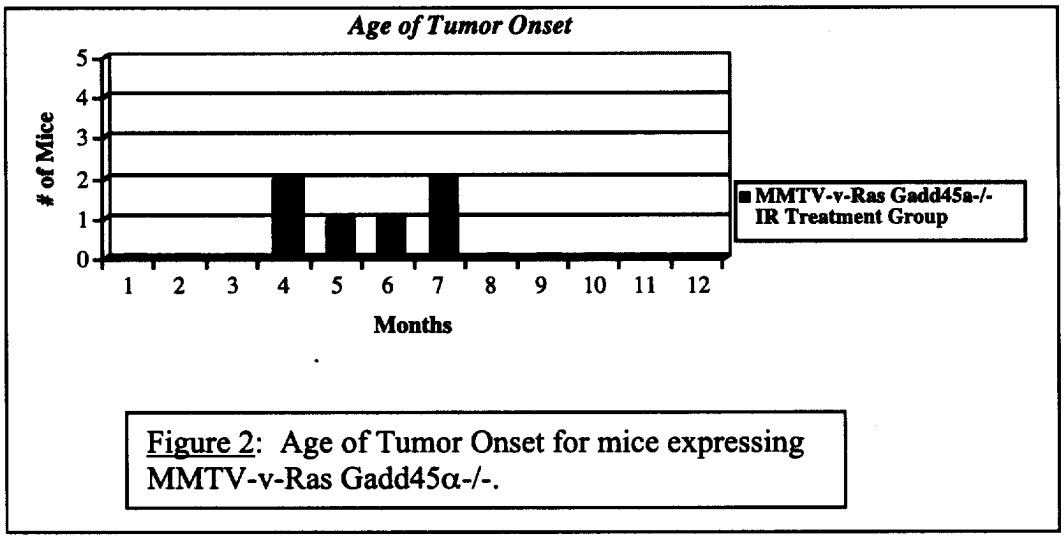

Figure 2: Age of Tumor Onset for mice expressing MMTV-v-Ras Gadd45α-/-.

Another interesting observation is the increase in the number of tumors per mouse in the MMTV-v-Ras Gadd45α-/- and MMTV-v-Ras Gadd45α+/- treatment groups compared to MMTV-v-Ras Gadd45α+/+ group. All MMTV-v-Ras Gadd45α+/+ mice that have developed masses have only developed one mass per mouse. On the other hand, the other two treatment groups have had a number of mice develop multiple masses per mouse.

Our primary focus for the future for this research is now generating mice to fill the treatment groups and monitoring them for tumor formation. Due to a shortage of Gadd45α/β-/- mice, the MMTV-v-Ras and MMTV-c-Myc Gadd45α/β -/- and control mice are lagging. We plan to have these treatment groups filled within the next year. After the experimental groups have been filled, more careful evaluation of the data, including statistical significance calculations, will be performed.

For Task IIB, upon removal of the mammary tumor, it is fixed in 10% Buffered Formalde-Fresh. We are in the process of sending out these samples for histological analysis.

For Task IIC, upon removal of mammary tumors from selected mice, cell cultures are being established to examine the cellular and molecular characteristics of the breast cancer cells. Currently, we have developed on tumor cell line from a mammary tumor from a MMTV-v-Ras Gadd45α-/- mouse. In the near future, we will begin growth protocols, FACS analysis for cell cycle and examining secondary genetic mutations.

KEY RESEARCH ACCOMPLISHMENTS

* Generating MMTV-ras Gadd45α-/- mice.
* Generating MMTV-myc Gadd45α-/- mice.
* Generating proper control mice (i.e. MMTV-v-Ras or MMTV-c-Myc and Gadd45α+/+ or Gadd45α+/- and Gadd45α/β+/+ or Gadd45α/β+/-).
* Establishing experimental treatment groups.
* Development of tumor cell line.

REPORTABLE OUTCOMES

Breast tumorigenesis seems to be accelerated in the MMTV-v-Ras Gadd45α-/- group when compared to the MMTV-v-Ras Gadd45α+/+ group. There is an increase in the number of tumors per mouse in the MMTV-v-Ras Gadd45α-/- and MMTV-v-Ras Gadd45α+/- treatment groups compared to MMTV-v-Ras Gadd45α+/+ group. All MMTV-v-Ras Gadd45α+/+ mice that have developed masses have only developed one mass per mouse, whereas the other two treatment groups have had a number of mice develop multiple masses per mouse.

CONCLUSIONS:

Summary of Results: Breast tumorigenesis seems to be accelerated in the MMTV-v-Ras Gadd45α-/- group when compared to the MMTV-v-Ras Gadd45α+/+ group. Tumors begin to appear in the MMTV-v-Ras Gadd45α-/- mice as early as 2 months of age, whereas the wildtype mice do not seem to develop tumors until 5 months of age. Interestingly, Gadd45α+/- mice begin to develop tumors at 4 months of age, which suggests haplo-deficiency of Gadd45α is sufficient for acceleration of mammary tumor development. Tumorigenesis seems to be accelerated in the MMTV-v-Ras Gadd45α-/- irradiated mice when compared to unirradiated mice. Another interesting observation is the increase in the number of tumors per mouse in the MMTV-v-Ras Gadd45α-/- and MMTV-v-Ras Gadd45α+/- treatment groups compared to MMTV-v-Ras Gadd45α+/+ group. All MMTV-v-Ras Gadd45α+/+ mice that have developed masses have only developed one mass per mouse. On the other hand, the other two treatment groups have had a number of mice develop multiple masses per mouse.

Recommended Changes for Future Work: Over the past year of the granting period, we have realized the necessity for revising the original SOW and have therefore submitted an official change in the SOW. Please find a copy of the revised SOW in Appendix 1 (Pending approval).

Overall Significance of the Research: The overall objective of the proposed research is to investigate the role of the Gadd45 family of genes in breast carcinogenesis. Gadd45 proteins have been shown to play an important role in cell cycle control and response to anti-cancer agents. Results obtained should provide information that will increase the understanding of the molecular basis of breast cancer development and may be utilized to design rational, novel therapies for treatment of breast cancer.

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Revised Statement of Work
Army Award DAMD17-02-1-0575
PI – Dan A. Liebermann

Task 1. Assess the effect of Gadd45a or Gadd45a/b deficiencies on oncogene driven breast carcinogenesis.

A. Establishment of MMTV-v-Ras and MMTV-c-Myc mice that are deficient for Gadd45a or Gadd45a/b will be accomplished by matting Gadd45a-/- & Gad45a/b-/- knockout mice with MMTV-v-Ras and MMTV-c-Myc. The proper control mice will also be established by mating MMTV-v-Ras and MMTV-c-Myc mice with Gadd45+/+ and Gadd45+/- mice. **(Months 1-14)**

B. Assess the effect of Gadd45a & Gad45a/b deficiencies on breast cancer development in MMTV-v-Ras and MMTV-c-Myc mice that are either wild-type, heterozygous or deficient for Gadd45a or Gadd45a/b expression. **(Months 14-30)**

1. Determine mean age of breast tumor onset & incidence of tumor development in Gadd45a-/- & a/b-/- mice compared to both Gadd45+/+ and Gadd45+/- mice
2. Determine tumorigenic state & histological analysis of tumors.
3. Determine aggressiveness of the tumorigenic phenotype by incidence of lung metastasis in tumor bearing mice.

C. Assess the effect of Gadd45a or Gadd45a/b deficiencies on breast cancer development in MMTV-v-Ras or MMTV-c-Myc mice that are deficient for Gadd45a or Gadd45a/b following hormone stimulation, IR treatment or DMBA treatment as delineated in Task1B. **(Months 20-30)**

Task 2. Characterization of breast tumors promoted by Gadd45 deficiency and oncogenic v-Ras or c-Myc.

A. Establish and characterize tumor cell lines from primary tumors arising from mice that express either v-Ras or c-Myc and are deficient for Gadd45a or Gadd45a/b. **(Months 24-36)**

1. Determine growth properties, & cell cycle kinetics of early passage tumor cells.
2. Assess genomic stability in tumor cells by karyotype analysis.
3. Assess activation of cell cycle checkpoints following exposure of the cells to genotoxic stress, using FACS analysis.
4. Determine responsiveness of the tumor cell to apoptotic stimuli (IR, UV), using a clonogenic survival assay and direct analysis of apoptoic cell death determined by Annexin & TUNEL staining
5. Determine ability of cells to repair DNA using the unscheduled DNA synthesis (UDS) assay.
6. Determine nature of secondary genetic events in mammary tumors arising as the result of Gadd45 deficiencies:

a) Assess genetic aberration in c-Myc, H-ras, erb-2, p53, BRCA1/2, p21 &/or wild-type Gadd45 using, FISH analysis, Western blotting, RT-PCR & DNA sequencing, and the protein truncation test.
b) Use cDNA microarrays as a more global approach to identify secondary genetic lesions that may contribute to accelerated mammary tumor development in Gadd45a-/- & a/b-/-mice.